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Binary regulation of interleukin(IL)-6 production by EP_1 and EP_2/EP_4 subtypes of PGE_2 receptors in IL-1β-stimulated human gingival fibroblasts

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Prostaglandin E_2 (PGE_2) exerts its biological actions via EP receptors, which are divided into four subtypes of EP_1, EP_2, EP_3 and EP_4. In the present study, we investigated whether PGE_2 regulated interleukin(IL)-6 production in human gingival fibroblasts (HGF) stimulated with IL-1β and if so, which subtype(s) of PGE_2 receptors were involved. Indomethacin, a cyclooxygenase inhibitor, significantly enhanced IL-1β-induced IL-6 production by HGF, although it completely inhibited IL-1β-induced PGE_2 production. Exogenous PGE_2 suppressed the IL-1β-induced IL-6 production. Reverse transcription-polymerase chain reaction analysis demonstrated that mRNA of EP_1, EP_2 and EP_4, but not EP_3 mRNA, was expressed in unstimulated and IL-1β-stimulated HGF. 11-deoxy-PGE_1, a selective $EP_2/EP_3/EP_4$ agonist, and butaprost, a selective EP_2 agonist, inhibited IL-1β-induced IL-6 production, although butaprost was less potent than 11-deoxy-PGE_1. 17-phenyl-ω-trinor PGE_2, an EP_1 agonist, enhanced IL-1β-induced IL-6 production. Based on these data, we suggest that PGE_2 can up- or downregulate IL-1β-induced IL-6 production via EP_1 receptors or via EP_2/EP_4 receptors in HGF, respectively. Expression and function of EP_1, EP_2 and EP_4 receptors in HGF may play critical roles in controlling inflammatory periodontal conditions.

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Prostaglandin (PG) E_2 has diverse activities including vasodilation, inhibition of lymphocyte proliferation and bone resorption (1). The diversity and specificity of the cellular effects of PGE_2 are believed to be dependent on activation of multiple functionally distinct subtypes of PGE_2 receptors expressed on the cell surface (2, 3). Based on their ligand binding selectivities and activation mechanisms of signaling pathways, the subtypes of PGE_2 receptors are classified into EP_1, EP_2, EP_3 and EP_4 (2, 3). EP_1 receptors mediate increase in intracellular calcium levels (4). EP_2 and EP_4 receptors activate adenylate cyclases via a cholera toxin-sensitive, stimulatory G protein and elevate intracellular cAMP levels (5–7). EP_2 receptors are sensitive to butaprost, an agent that selectively binds PGE_2 receptors, whereas EP_4 receptors are not (7, 8). Multiple isoforms of EP_3 receptors with different C-terminal tails are generated by alternative mRNA splicing (9). EP_3 receptor variants mediate several signaling pathways including inhibition and stimulation of adenylate cyclase, activation of phospholipase C and mobilization of intracellular calcium (10, 11).

Numerous studies have demonstrated that PGs including PGE_2 are involved in the pathogenesis of periodontal disease (12). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit PG synthesis, demonstrate an ability to prevent periodontal destruction (13, 14). PGE_2 levels are reportedly enhanced in inflamed human gingiva, compared to normal gingiva (15). Furthermore, PGE_2 has been shown to be a potent stimulator of bone resorption (16, 17) and to be associated with attachment loss (12, 18).

Interleukin(IL)-6 is a pleiotropic cytokine produced by a variety of cells including monocytes/macrophages, activated T cells, endothelial cells and fibroblasts (19, 20). IL-6 promotes B cell activation and induces hepatocytes to produce acute phase proteins (19, 20). Furthermore, IL-6 stimulates induction of osteoclast formation and bone resorption (21). It has been reported that IL-6 levels in gingival crevicular fluid are correlated with bleeding index and probing depth in patients with adult periodontitis (22). In inflamed human gingival tissues, IL-6 mRNA and protein have been detected in several types of cells including gingival fibroblasts (23, 24). *In vitro* studies have shown that human gingival fibroblasts (HGF) synthesize IL-6 *de novo* in response to IL-1β, tumor necrosis factor α (TNF α) and lipopolysaccharides (LPS) (25). Therefore, it is likely that IL-6 production by HGF is involved in the destruction of periodontal tissue. Czuszak *et al.* (26) have reported that PGE_2 potentiates IL-1β-induced IL-6 production in HGF derived from severe adult periodontitis patients. On the other hand, Takigawa *et al.* (27) have shown that PGE_2 suppresses IL-1β-induced IL-6 production in HGF derived from periodontally healthy subjects. These findings suggest that PGE_2 may play an important role in controlling IL-6 generation in periodontal lesions. However, the mechanism by which PGE_2 regulates IL-1β-induced IL-6 production in HGF remains unclear.

In the present study, we investigated the effect of PGE_2 on IL-1β-induced IL-6 production in HGF derived from periodontally healthy subjects and examined which subtypes of PGE_2 receptors were involved in the regulation of IL-6 production.

Material and methods

Cell preparation

HGF were obtained from gingiva of premolars or third molars extracted from three periodontally healthy subjects. Our study protocol, including biopsy of gingival tissue, satisfied the ethical standards of Tokyo Medical Dental University and informed consent was obtained from all the subjects. Fibroblasts which extended from pieces of gingiva were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) (Bioserum, Victoria, Australia) in the presence of 100 U/ml of penicillin (Sigma Chemical Co., St Louis, MO, USA) and 100 μg/ml of streptomycin (Sigma Chemical Co.) in a humidified atmosphere of 5% CO_2 at 37°C. The cells used for experiments were between the fifth and fifteenth passages.

Cell stimulation

HGF were seeded into 96-well plates at a concentration of 5×10^4 cells/ml. When the cells reached confluence, the medium was changed to α-MEM containing 0.5% FBS to minimize any serum-induced effects on HGF. After 24 h, the cells were stimulated with vehicle or 2 ng/ml of human recombinant IL-1β (Sigma Chemical Co.) in the presence or absence of indomethacin (Wako, Tokyo, Japan), PGE_2 (Cascade Biochem LTD, Berkshire, UK), 17-phenyl-ω-trinor PGE_2 (Cayman Chemical, Ann Arbor, MI, USA), 11-deoxy-PGE_1 (Cayman Chemical), butaprost (a gift from ONO Pharmaceutical Co. Ltd, Tokyo, Japan), ONO-AP-324 (an EP_3 agonist, a gift from ONO Pharmaceutical Co. Ltd), dibutyryl cAMP (Wako) and forskolin (Wako) in the indicated combination and concentrations, which were added to the cells 30 min prior to stimulation with IL-1β.

Reverse transcription-polymerase chain reaction (RT-PCR)

HGF were treated with 2 ng/ml of IL-1β for the indicated periods. The medium was removed and total cellular RNA was isolated. In brief, the cells were lysed and RNA was extracted by the guanidinium thiocyanate/phenol/chloroform method, using ISOGEN (Nippon Gene, Toyama, Japan). After extracting total RNA from HGF, cDNAs were synthesized from 2 μg of total RNA with RAV2 reverse transcriptase and oligo(dT)primers (Takara Co., Shiga, Japan), as described previously (28). The specific primer pairs for human EP_1, EP_2, EP_3, EP_4 and β-actin were selected, according to the cDNA sequences reported by Funk *et al.* (29), Regan *et al.* (5), Adam *et al.* (30), Bastien *et al.* (31) and Ponte *et al.* (32), respectively. The primers were EP_1: sense primer, 5′-TCTACCTCCCTGCAGCGGCCACTG-3′, antisense primer, 5′-GAAGTGGCTGAGGCCGCTGTGCCGGGA-3′; EP_2: sense primer, 5′-TTCATCCGGCACGGGCGGACCGC-3′, antisense primer, 5′-GTCAGCCTGTTTACTGGCATCTG-3′; EP_3: sense primer,

5′-GAGCACTGCAAGACACACACGGAG-3′, antisense primer, 5′-GATCTCCCATGGGTATTACTGACAA-3′; EP_4: sense primer, 5′-CCTCCTGAGAAAGACAGTGTC-3′, antisense primer, 5′-AGGACTCAGAGAGTGTCTT-3′; and β-actin: sense primer, 5′-GTG GGCATGGGTCATCAGAAGGAT-3′, antisense primer, CTCCTTAATGTCACGCACGATTTC-3′. The PCR reaction was performed using 25 μM of each primer, 2.5 mM of each dNTP and 2.5 units of Taq DNA polymerase (Takara Co.) in an automated DNA thermal cycler (Takara Co.). The PCR amplification was comprised of 35 cycles (EP_1, EP_2, EP_3 and EP_4) or 25 cycles (β-actin) of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 3 min. The RT-PCR products were resolved by electrophoresis in 2% agarose gels and stained with ethidium bromide. The identity of the RT-PCR products of EP_1, EP_2, EP_3 and EP_4 was confirmed by restriction endonuclease digestion and nucleotide sequence analysis (data not shown).

PGE_2 assay

The amounts of PGE_2 in the conditioned media collected from control or IL-1β-stimulated cells were determined by a commercially available enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Assay of IL-6

IL-6 levels in the conditioned media collected from control and IL-1β-stimulated cells were determined by a commercially available ELISA kit (Endogen Inc., Woburn, MA, USA).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Data were subjected to 1-way analysis of variance (ANOVA), using the StatView 4.0 program on a Macintosh computer. Fisher's protected least significance test was used in the post hoc comparison of specific groups.

Results

Effect of indomethacin on IL-1β-induced IL-6 and PGE_2 production in HGF

To examine whether PGs endogenously produced affected IL-6 production in IL-1β-stimulated HGF, the effect of indomethacin, a cyclooxygenase inhibitor, on IL-6 production was investigated in the cells. Indomethacin significantly increased IL-1β-induced IL-6 production ($p<0.001$), although PGE_2 production by IL-1β was completely inhibited by indomethacin (Figs 1A and 1B), which suggests that endogenous PGs were involved in downregulation of IL-1β-induced IL-6 production.

Effect of PGE_2 on IL-6 production in HGF

The effect of exogenous PGE_2 on IL-1β-induced IL-6 generation was investigated, as IL-1β-stimulated HGF primarily produce PGE_2 (33). HGF was treated with indomethacin to exclude involvement of endogenous PGE_2 prior to addition of PGE_2. Exogenous PGE_2 suppressed IL-1β-induced IL-6 production in a concentration-dependent fashion (Fig. 2A). PGE_2 alone induced an increase of IL-6 production, but the levels of

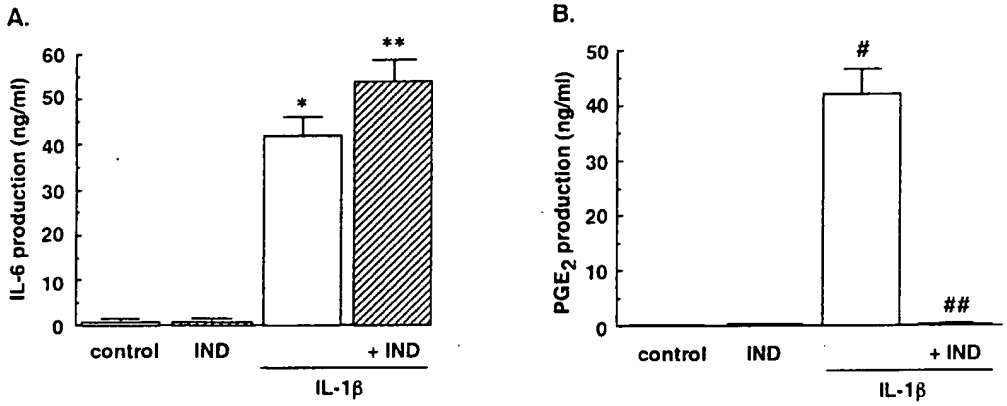

Fig. 1. Effect of indomethacin on production of IL-6 (A) and PGE_2 (B) in IL-1β-stimulated HGF. HGF was stimulated with vehicle or 2 ng/ml of IL-1β in the presence or absence of 1 μM of indomethacin (IND) for 24 h. After incubation, IL-6 levels (A) and PGE_2 levels (B) in the culture media were measured by ELISA, as described in Material and methods. Values are mean ± SD ($n=4$). Data are representative of three separate experiments. *Significantly different from control ($p<0.0001$). **Significantly different from IL-1β alone ($p<0.001$). #Significantly different from control ($p<0.0001$). ##Significantly different from IL-1β alone ($p<0.0001$).

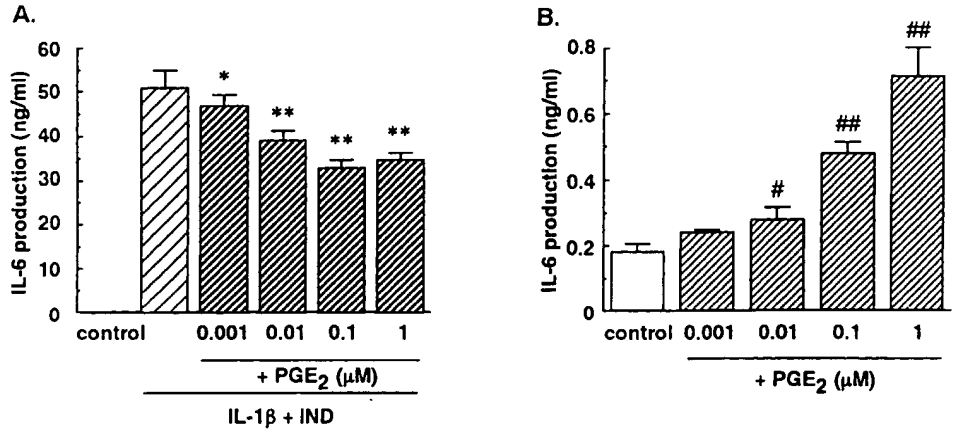

Fig. 2. Effect of exogenous PGE_2 on IL-6 production in HGF. HGF was treated with vehicle or 2 ng/ml of IL-1β in the presence of 1 μM of indomethacin and various doses of PGE_2 (A) or with vehicle or various doses of PGE_2 alone (B) for 24 h. After incubation, IL-6 levels in the culture media were measured by ELISA, as described in Material and methods. Values are mean $\pm$ SD ($n=4$). Data are representative of three separate experiments. *Significantly different from IL-1β + IND ($p<0.05$). **Significantly different from IL-1β + IND ($p<0.0001$). #Significantly different from control ($p<0.05$). ##Significantly different from control ($p<0.0001$).

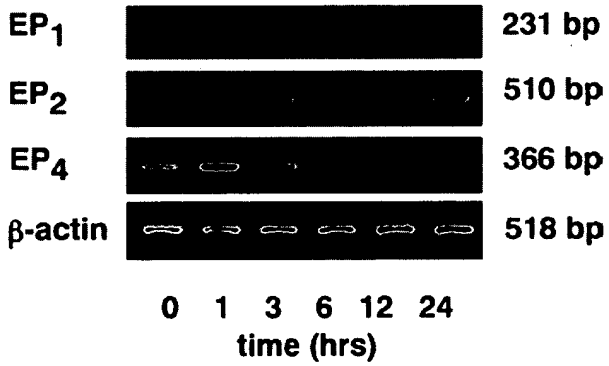

Fig. 3. Detection of mRNA expression of EP_1, EP_2 and EP_4 receptors in HGF. HGF was stimulated with 2 ng/ml of IL-1β for the indicated periods. Total RNA was extracted from the cells and RT-PCR was performed to detect mRNA expression of EP_1, EP_2 and EP_4 receptors and β-actin in HGF, as described in Material and methods.

IL-6 induced by PGE_2 were very small, compared with those of IL-6 induced by IL-1β (Figs. 2A and 2B).

Detection of EP receptor mRNA in HGF

To examine the involvement of EP receptors (EP_1, EP_2, EP_3, EP_4) in PGE_2 regulation of IL-6 production, mRNA expression of the subtypes was examined in unstimulated and IL-1β-stimulated HGF by RT-PCR. mRNA of EP_1, EP_2 and EP_4 receptors was expressed in unstimulated and IL-1β-stimulated HGF (Fig. 3). Although RT-PCR performed in the present study was not quantitative, IL-1β appears to have induced mRNA expression of EP_2 and EP_4 receptors in HGF (Fig. 3). EP_3 mRNA expression was not detected in unstimulated and IL-1β-stimulated HGF (data not shown).

Effect of various EP receptor agonists on IL-1β-induced IL-6 production

We evaluated which subtypes of EP receptors were involved in IL-1β-induced IL-6 production, using various selective EP receptor agonists. Treatment of IL-1β-stimulated HGF with a selective EP_2/EP_3/EP_4 agonist, 11-deoxy-PGE_1, inhibited IL-6 production in a concentration-dependent manner (Fig. 4A). Butaprost, a selective EP_2 agonist, inhibited IL-1β-induced IL-6 production in a concentration-dependent fashion, although the effect of butaprost was less potent than that of 11-deoxy-PGE_1 (Fig. 4B). Next, the effect of 17-phenyl-ω-trinor PGE_2, a selective EP_1 agonist, on IL-6 production by IL-1β-stimulated HGF was examined. 17-phenyl-ω-trinor PGE_2 enhanced IL-1β-induced IL-6 production by HGF (Fig. 4C). ONO-AP-324, a selective EP_3 agonist, had no effect on IL-1β-induced IL-6 production (data not shown).

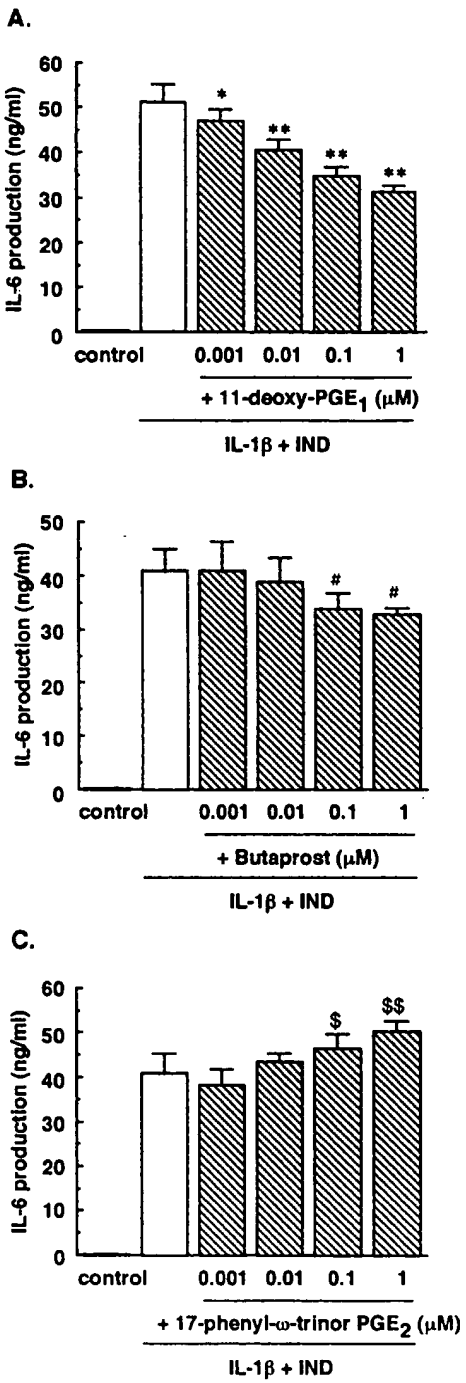

Fig. 4. Effect of 11-deoxy PGE_1, butaprost and 17-phenyl-ω-trinor PGE_2 on IL-1β-induced IL-6 production in HGF. HGF was stimulated with 2 ng/ml of IL-1β in the presence of 1 μM of indomethacin (IND) with or without various doses of 11-deoxy-PGE_1 (A), butaprost (B) and 17-phenyl-ω-trinor PGE_2 (C). After 24 h, IL-6 levels in the culture media were measured by ELISA, as described in Material and methods. Values are mean $\pm$ SD ($n=4$). Data are representative of three separate experiments. *Significantly different from IL-1β + IND ($p<0.05$). **Significantly different from IL-1β + IND ($p<0.0001$). #Significantly different from IL-1β + IND ($p<0.05$). $Significantly different from IL-1β + IND ($p<0.05$). $$Significantly different from IL-1β + IND ($p<0.005$).

Effect of dibutyryl cAMP and forskolin on IL-1β-induced IL-6 production

Since EP_2 and EP_4 receptors are involved in elevation of intracellular cAMP levels (5–7), the effects of dibutyryl cAMP, a cAMP analog, and forskolin, an activator of adenylate cyclases, on IL-6 production by IL-1β-stimulated HGF were investigated. As shown in Figure 5, dibutyryl cAMP

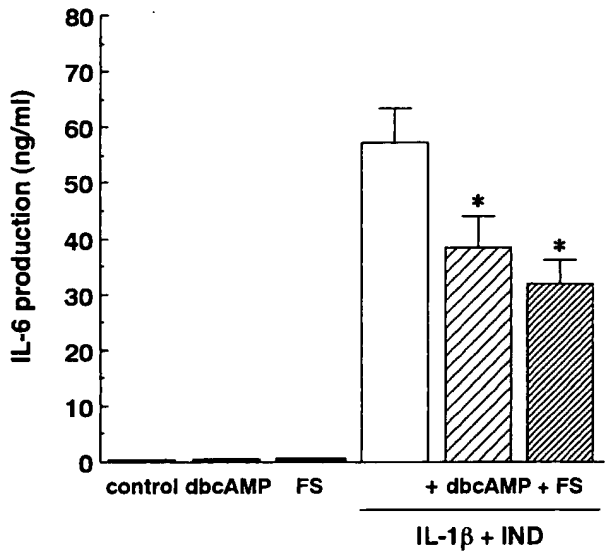

Fig. 5. Effect of dibutyryl cAMP and forskolin on IL-1β-elicited IL-6 production in HGF. HGF were stimulated with 2 ng/ml of IL-1β in the presence of 1 μM of indomethacin (IND) with or without 100 μM of dibutyryl cAMP (dbcAMP) and 10 μM of forskolin (FS). After 24 h, IL-6 levels in the culture media were measured by ELISA, as described in Material and methods. Values are mean $\pm$ SD ($n=4$). Data are representative of three separate experiments. *Significantly different from IL-1β + IND ($p<0.0001$).

and forskolin significantly inhibited IL-1β-induced IL-6 production ($p<0.0001$).

Discussion

In the present study, we demonstrated that PGE_2 suppressed IL-1β-induced IL-6 production in HGF derived from periodontally healthy subjects. Treatment of the IL-1β-stimulated HGF with indomethacin, a cyclooxygenase inhibitor, significantly enhanced IL-6 production, although IL-1β-induced PGE_2 production was completely inhibited (Figs 1A and 1B). Furthermore, exogenous PGE_2 attenuated IL-1β-induced IL-6 production in a dose-dependent manner (Fig. 2A). These data suggest that PGE_2 endogenously produced is involved in regulation of IL-6 production in IL-1β-stimulated HGF, which is consistent with the report by Takigawa *et al.* (27).

The diversity and selectivity of the effects of PGE_2 are dependent on expression of EP subtypes of PGE_2 receptors. It is known that EP_1 receptors and EP_2/EP_4 receptors are involved in increases in intracellular calcium levels and elevation of intracellular cAMP levels, respectively (4–7). We confirmed increases in intracellular Ca^{2+} levels and intracellular cAMP levels in PGE_2-treated HGF (data not shown), suggesting the presence of functional EP_1 and EP_2/EP_4 receptors in the cells. mRNA of EP_1, EP_2 and EP_4 receptors was expressed in unstimulated and IL-1β-stimulated HGF (Fig. 3). Although RT-PCR performed in the present study was not quantitative, it appears that IL-1β induced mRNA expression of EP_2 and EP_4 receptors in HGF (Fig. 3). It has been shown that EP_2 and EP_4 receptor expression is upregulated in murine RAW 264.7 macrophage cell lines after stimulation with LPS (34, 35). Furthermore, recent studies have demonstrated that EP_1, EP_3 and EP_4 receptors may exist in the nuclear envelope as well as in plasma membranes in endothelial cells (36, 37). Further studies are necessary to reveal the mechanism and localization of expression of EP_1, EP_2 and EP_4 receptors in HGF.

17-phenyl-ω-trinor PGE_2, a selective EP_1 agonist, upregulated IL-1β-elicited IL-6 production in HGF (Fig. 3C). EP_1 receptors are involved in increases of intracellular Ca^{2+} levels. In cloned osteoblast-like MC3T3-E1 cells, PGE_2 stimulated IL-6 synthesis through Ca^{2+} mobilization from extracellular space via EP_1 receptors (38). It has been demonstrated that Ca^{2+}-elevating agents increase expression of IL-6 gene in lymphocytes and skin fibroblasts (39). Recently, we have demonstrated that $PGF_{2\alpha}$ induces IL-6 production via intracellular Ca^{2+} mobilization in HGF (40). Thus, it is very likely that activation of EP_1 receptors causes increased IL-1β-elicited IL-6 production via Ca^{2+}-dependent pathways in HGF. On the other hand, 11-deoxy-PGE_1, a selective $EP_2/EP_3/EP_4$ agonist, and butaprost, a selective EP_2 agonist, inhibited IL-6 production by IL-1β-stimulated HGF. EP_2 and EP_4 receptors are linked to intracellular cAMP elevation (5–7). Dibutyryl cAMP and forskolin caused suppression of IL-1β-induced IL-6 production (Fig. 5). Therefore, it is very likely that cAMP-dependent pathways via EP_2/EP_4

receptors are involved in PGE_2 downregulation of IL-1β-induced IL-6 production in HGF. In the present study, the mechanism by which cAMP attenuated IL-1β-induced IL-6 production in HGF was not investigated. Transcriptional factors including nuclear factor(NF)-IL-6, NFκB and AP-1 are required for IL-6 gene activation (41) and cAMP inhibits NF-κB-mediated transcription in human monocytic cells and endothelial cells (42). Zitnik *et al.* (43) have showed that in human lung fibroblasts cAMP downregulates IL-1α-induced IL-6 production, which is mediated by decreased IL-6 gene expression. However, Takigawa *et al.* (27) have reported that IL-1β-induced IL-6 production in HGF may be inhibited by PGE_2 at the post-transcriptional level. Further studies are necessary to reveal the mechanism by which cAMP downregulates IL-1β-induced IL-6 production in HGF. Taken together, we suggest that PGE_2 can upregulate IL-1β-elicited IL-6 synthesis through Ca^{2+}-dependent pathways via EP_1 receptors or downregulate this synthesis through cAMP-dependent pathways via EP_2/EP_4 receptors in HGF. Interestingly, treatment of HGF with PGE_2 alone induced IL-6 production, although the levels of IL-6 induced by PGE_2 were very small, compared with those of IL-6 induced by IL-1β (Figs 2A and 2B). Treatment of HGF with 17-phenyl-ω-trinor PGE_2 or 11-deoxy-PGE_1 alone caused a slight increase of IL-6 production (data not shown). Recently, it has been demonstrated that cAMP induces IL-6 production in HGF (44). cAMP seems to differently function in regulating IL-6 production in unstimulated and IL-1β-stimulated HGF.

In addition to promoting B cell differentiation and T cell activation, IL-6 has been shown to be a potent bone-resorptive agent and an inhibitor of bone formation (21, 45). It has been reported that IL-6 levels in gingival crevicular fluid are correlated with bleeding index and probing depth in patients with adult periodontitis (22). In inflamed human gingival tissues, IL-6 mRNA and protein have been detected in several types of cells including gingival fibroblasts (23, 24). It is thought that IL-6 production by HGF is involved in the destruction of periodontal tissue. Czuszak *et al.* (26) have reported that PGE_2 potentiates IL-1β-induced IL-6 production in HGF derived from severe adult periodontitis patients. This is contrary to our present data and the report by Takigawa *et al.* (27) which have shown that PGE_2 inhibits IL-1β-induced IL-6 production in HGF derived from periodontally healthy subjects. The difference in regulation of IL-1β-induced IL-6 production by PGE_2 between these cells remains to be elucidated. However, it may be due to a difference in the extent of expression or function of EP_1 and EP_2/EP_4 receptors between the two cells. Our preliminary data showed that PGE_2 increased IL-1β-induced IL-6 production, with cAMP having no effect, in HGF of some severe periodontitis patients (data not shown). The pathophysiological relevance of binary regulation of IL-1β-induced IL-6 production by EP_1 and EP_2/EP_4 receptors is unclear. However, it is likely that the regulation of IL-1β-induced IL-6 production by PGE_2 is primarily mediated via EP_1 receptors in HGF from severe periodontitis patients and via EP_2/EP_4 receptors in HGF from periodontally healthy subjects. Further studies are needed to elucidate this.

In conclusion, we suggest that PGE_2 can upregulate IL-1β-elicited IL-6 synthesis through Ca^{2+}-dependent pathways via EP_1 receptors or downregulate this synthesis through cAMP-dependent pathways via EP_2/EP_4 receptors in HGF. Expression and function of EP_1, EP_2 and EP_4 receptors in HGF may play critical roles in controlling inflammatory periodontal conditions.

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